

## Dietary (n-3) fatty acids alter plasma fatty acids and leukotriene B synthesis by stimulated neutrophils from healthy geriatric Beagles

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### Abstract:

The study objective was to determine the effect of feeding food enriched in (n-3) fatty acids (FA) on plasma FA profiles and leukotriene B (LTB) synthesis by stimulated peripheral blood neutrophils from dogs. For 36 weeks, two groups of dogs (n = 5) were fed food that contained either a low ratio of (n-6)–(n-3) FA (1.31:1; fish oil-enriched food) or a high ratio of (n-6)–(n-3) FA (40.6:1; corn oil-enriched food). Consumption of food enriched in fish oil resulted in higher plasma concentrations of eicosapentaenoic acid and docosahexaenoic acid and lower concentrations of arachidonic acid. Neutrophils from dogs fed fish oil-enriched food produced 7.6-fold more LTB<sub>5</sub> (P = 0.002), and the ratio of LTB<sub>5</sub>–LTB<sub>4</sub> concentrations was 8.3-fold higher (P<0.001) compared with dogs fed corn oil-enriched food. Dietary FA can modulate leukotriene production by neutrophils in dogs, and suggests that foods enriched in (n-3) FA from fish oil may have value in the treatment of canine inflammatory diseases.

### Article:

#### 1. Introduction

A variety of diets and fatty acid (FA) supplements rich in (n-3) fatty acids (FAs) from fish oil are currently marketed for use in dogs. We have previously shown that healthy, geriatric Beagles fed foods enriched in (n-3) FAs from fish oil for 12 weeks have altered plasma FA profiles [1], similar to what has been reported in humans, horses, and laboratory animals [2–5]. In addition, a ratio of dietary (n-6)–(n-3) FAs of 1.4:1 depressed cell- mediated immunity,

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based on results of a delayed-type hypersensitivity (DTH) skin test, and decreased prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) production by stimulated mononuclear cells [1]. Altering the ratio of (n-6)–(n-3) FAs also affected T-cell subpopulations in aged dogs, in that after immunization with a novel protein these dogs had decreased numbers of CD4<sup>+</sup> lymphocytes and decreased CD4<sup>+</sup>-to-CD8<sup>+</sup> ratios [6]. Furthermore, we have shown that there is an interaction between dietary vitamin E and (n-3) FAs such that the effects of an optimum amount of dietary vitamin E concentration on the DTH response are blunted by dietary (n-3) FAs [7]. This study is a continuation of these investigations in aged Beagles, whereby we report the effect of consumption of a diet enriched in (n-3) FAs for 36 weeks on plasma FA profiles and on leukotriene (LT) B<sub>4</sub> and LTB<sub>5</sub> production by peripheral blood neutrophils.

The type of LT that cells produce, and consequently, communication between cells of the immune system, can potentially be modulated through dietary supplementation of (n-3) FAs [2,8–10]. When immune cells are activated by a chemical or physical insult, FAs are mobilized from cell membrane phospholipids and metabolized into eicosanoids [11]. Leukotrienes are eicosanoids with chemotactic properties; they are also involved in regulation of inflammatory and immune processes. Arachidonic acid [AA; 20:4(n-6)] is a 20 carbon (n-6) polyunsaturated FA that is incorporated into cell membrane phospholipids. In neutrophil cell membranes, AA is metabolized by 5-lipoxygenase to yield LTA<sub>4</sub>, which is converted to LTB<sub>4</sub> by LTA<sub>4</sub>-hydrolase. Production of LTB<sub>4</sub> by stimulated peripheral blood neutrophils reflects the plasma concentration of AA, the FA from which LTB<sub>4</sub> is derived [3]. Leukotrienes of the 4-series regulate inflammatory cytokine production [8]. The types of leukotrienes produced from eicosapentaenoic acid [EPA; 20:5 (n-3)], a 20 carbon (n-3) polyunsaturated FA, are less biologically potent than analogues synthesized from AA [8]. For example, LTB<sub>5</sub> which is produced by metabolism of EPA, is 10-fold less potent as a neutrophil chemoattractant than LTB<sub>4</sub> [12,13].

The goal of this investigation was to determine the effect of feeding different amounts of (n-3) FAs on plasma FA profiles and leukotriene production by peripheral blood neutrophils from dogs. To achieve this goal, we measured LTB<sub>4</sub> and LTB<sub>5</sub> production by calcium-ionophore stimulated peripheral blood neutrophils of dogs fed either corn oil- or fish oil- enriched foods.

## **2. *Materials and methods***

### **2.1. *Animals***

Ten healthy, female, geriatric (7- to 10-year-old) Beagles that weighed between 7.6 and 13.1 kg were selected for this study (Hill's Pet Nutrition Inc., Topeka, KS, USA).

All dogs had been previously vaccinated against canine distemper, parvovirus, and rabies according to standard protocols, and none had chronic systemic disease, as determined on the basis of results of physical examination, complete blood count (CBC) determination, serum biochemical analyses, urinalysis, and fecal examination for parasites. Dogs were housed in pairs of two in indoor runs and fed once daily in the morning. Dogs experienced enrichment through interactions with each other and with the caretakers. Four

additional healthy Beagles used in the teaching program at the College of Veterinary Medicine, Oregon State University, Corvallis, OR, USA were also included in the study. The experimental protocol was reviewed and approved by the Oregon State University Animal Care and Use Committee in accordance with principles outlined by the National Institutes of Health [14].

## 2.2. Foods

For 90 days before the study, the experimental dogs consumed a commercial food (Science Diet Canine Maintenance, Hill's Pet Nutrition Inc.) without enhancement of (n-6) and (n-3) FAs. The ratio of (n-6)–(n-3) FAs was 18:1 (Table 1). The source of (n-3) FAs in this food was 90% plant-derived from  $\alpha$ -linolenic acid (soybean oil). The healthy Beagles used in the teaching program also consumed the same baseline food (Science Diet Canine Maintenance, Hill's Pet Nutrition Inc.) as the experimental dogs, before the latter were switched to their respective experimental foods.

The experimental foods were prepared by a commercial company (Hill's Pet Nutrition Inc.). Experimental foods varied in the amount of (n-3) FAs they contained. The low (n-3) FA food contained a minimal amount of (n-3) FAs (0.5g [n-3] FAs/kg of food, wet-weight basis) and, thus, had a high ratio of (n-6)–(n-3) FAs (40.6:1). The high (n-3) FA food contained a larger amount of (n-3) FAs from fish oil (5.9 g [n-3] FAs/kg of food, wet-weight basis) and had a low ratio of (n-6)–(n-3) FAs (1.31:1). The amount of 18:3(n-3) FA ( $\alpha$ -linolenic acid) was low in both foods and roughly equivalent. Both foods contained 101 mg/kg of all-rac- $\alpha$ -tocopherol acetate. The basal food ingredients (by weight) included 54.8% water, 20.3% turkey, 15.0% corn, 4.5% pork liver, 2.0% soy meal, 1.0% beet pulp and 0.4% vitamin and mineral premixes. Rice hulls were used as the carrier for the vitamin premix, which contained 25 mg/kg cholecalciferol, 7500 mg/kg nicotinic acid, 5000 mg/kg calcium D-pantothenate, 21,800mg/kg thiamine mono- nitrate, 1250 mg/kg riboflavin, 2430 mg/kg pyridoxine hydrochloride, 250 mg/kg folic acid, 50 mg/kg biotin and 50mg/kg vitamin B-12. Calcium carbonate was used as the carrier for the mineral mix, which contained 80 g/kg zinc as zinc oxide, 6.0 g/kg manganese as manganese oxide, 280 g/kg iodine as calcium iodate, 1.0 g/kg cobalt as cobalt carbonate, 180 mg/kg selenium as selenium selenite, and 2.5 g/kg copper as copper chloride. The remaining 2% of the food was provided as added oil. The source of oil for the (n-3) enriched food was Menhaden fish oil (Menhaden fish oil, Zapata Protein, Reedville, VA, USA), whereas the source for the (n-6) enriched food was corn oil (Mazola corn oil, Mazola, Englewood Cliffs, NJ, USA). Foods were analyzed at a commercial laboratory (Woodson-Tenent Laboratories, Des Moines, IA, USA). Nutrient composition (by weight) was 77.4% moisture, 5.8% protein, 4.5% fat, 1.3% ash and 0.7% crude fiber and 10.3% carbohydrate. FA composition of the two experimental foods was determined (Table 1).

## 2.3. Study design

Dogs were ranked on the basis of body weight and assigned to two groups of five dogs each, such that body

Table 1  
Composition of selected fatty acids in foods fed to geriatric Beagles

Fatty acid	Baseline food without enhancement of (n-6) and (n-3) fatty acid content (g/kg of food)	Corn oil-enriched food with low (n-3) fatty acid content (g/kg of food)	Fish oil-enriched food with high (n-3) fatty acid content (g/kg of food)
14:0	0.2	0.2	1.4
16:0	6.9	7.5	7.1
18:0	3.3	2.5	2.3
Sum of SFA <sup>‡</sup>	10.4	10.2	10.9
16:1(n-7)	0.9	1.1	2.5
18:1(n-9)c	12.4	14.5	9.0
Sum of MUFA <sup>§</sup>	13.6	15.8	11.8
18:2(n-6)	7.2	19.9	7.2
18:3(n-3)	0.3	0.5	0.5
18:4(n-3)	<0.1	<0.1	0.6
20:4(n-6)	0.1	0.4	0.5
20:4(n-3)	<0.1	<0.1	0.3
20:5(n-3)	<0.1	<0.1	1.9
22:5(n-3)	<0.1	<0.1	0.4
22:6(n-3)	<0.1	<0.1	2.2
Sum of PUFA <sup>§</sup>	8.0	20.8	13.3
Sum of (n-6) fatty acids	7.5	20.3	7.7
Sum of (n-3) fatty acids	0.4	0.5	5.9
Ratio (n-6)–(n-3)	18.0:1	40.6:1	1.31:1

<sup>‡</sup>Sum of the saturated fatty acids (SFA) was determined as follows: 8:0+ 10:0+ 11:0+ 12:0+ 14:0+ 15:0+ 16:0+ 17:0+ 18:0+20:0+22:0 +24:0.

<sup>§</sup>Sum of the monounsaturated fatty acids (MUFA) was determined as follows: 14:1 + 15:1 + 16:1(n-7) + 17:1 + 18:1(n-9)c+ 18:1(n-7)+ 18:1 (n-9)t+ 20:1(n-9) + 22:1(n-9) + 24:1.

<sup>§</sup>Sum of the polyunsaturated fatty acids (PUFA) was determined as follows: 18:2(n-6)+18:3(n-6)+18:3(n-3)+18:4(n-3)+20:2(n-6)+20:3 (n-6) + 20:3(n-3) + 20:4(n-6) + 20:4(n-3) + 20:5(n-3) + 21:5(n-3) + 22:2(n-6) + 22:4(n-6) + 22:5(n-6) + 22:5(n-3) + 22:6(n-3).

weights were evenly distributed between the groups. Body weights were measured before the study began and weekly during the study. Food intake was quantitated and adjusted such that dogs did not gain or lose weight. Blood samples were collected after the experimental dogs had consumed their respective foods for 36 weeks. Blood samples were collected from the healthy Beagles used in the teaching program after they had consumed the baseline food for 4 weeks. Blood samples were collected before the daily meal offering into evacuated tubes containing EDTA (final concentration, 1.5 g/L), and plasma was harvested.

## 2.4. Plasma FAs

FA content of plasma samples was determined by use of gas chromatography, as previously described [15], using heptadecanoic acid as the internal standard. FA concentration was expressed as g/100g of FAs.

## 2.5. Leukotriene B4 and B5 quantification

Neutrophils were isolated and purified as previously described [16] from blood collected at 36 weeks for the experimental dogs and at 4 weeks for the healthy Beagles used in the teaching program. Aliquots of  $1 \times 10^6$  neutrophils were transferred to test tubes and the volume adjusted to 495  $\mu$ L with Hanks balanced salt solution containing 0.8 mmol/L

CaCl<sub>2</sub>. Neutrophils were then stimulated with 5 µL of calcium ionophore A23187 (Sigma Chemical Co., St. Louis, MO, USA) in 0.2% dimethyl sulfoxide (Syntex, West Des Moines, IA, USA) such that the final concentration of A23187 was 10 mmol/L, while unstimulated neutrophils received 5 µL of 0.2% dimethyl sulfoxide without calcium ionophore. All tubes were incubated for 5 min at 37°C as previously described [16]. Supernatants were stored at -70°C until subsequent LTB<sub>4</sub> and LTB<sub>5</sub> measurements were made. LTB<sub>4</sub> and LTB<sub>5</sub> were extracted, separated, and quantified as previously described [16]. Standard calibration curves for LTB<sub>4</sub> and LTB<sub>5</sub> were made by adding 100 ng of PGB<sub>3</sub> as an internal standard to samples containing 0.47–40 ng of LTB<sub>4</sub> and LTB<sub>5</sub> (Sigma). PGB<sub>3</sub> was chosen as the internal standard because it was widely separated from LTB<sub>4</sub> present in actual samples during HPLC separation, whereas PGB<sub>2</sub> coeluted with LTB<sub>5</sub> and PGB<sub>1</sub> coeluted with LTB<sub>4</sub> [17]. Concentrations of leukotrienes in test samples were calculated with reference to the standard curves. Final LTB<sub>4</sub> and LTB<sub>5</sub> concentrations were reported as nanograms of LTB<sub>4</sub> and LTB<sub>5</sub> per 1 x 10<sup>6</sup> cells.

## 2.6. Statistical analysis

Data are reported as means ± SEM. Using the Kolmogorov–Smirnov test, data that were found to be normally distributed were analyzed using analysis of variance. On the basis of the Homogeneity of Variance test, data that were homoscedastic were analyzed using analysis of variance followed by post hoc separation of the means using the Tukey–Kramer Multiple-Comparison test. When the assumptions of normality and equal variance were suspect, data were analyzed by the nonparametric Kruskal–Wallis Test, followed by post hoc separation of the means using the Kruskal–Wallis Z-Test. Correlations were evaluated with the Pearson correlation test. Overall significance was set at P < 0.05.

Statistical analyses were performed using the Number Cruncher Statistical System (NCSS) (Kaysville, UT, USA), version 2004 ([www.ncss.com](http://www.ncss.com)).

## 3. Results

### 3.1. Animals and foods

All dogs readily consumed their respective FA- enriched foods. At the beginning of the study dogs consuming the high (n-3) FA food weighed 10.0±0.7 kg (mean±SEM). At the end of the study these dogs weighed 10.8±0.7kg. At the beginning of the study the dogs consuming the low (n-3) FA food weighed 10.5 ± 0.8 kg (mean ± SEM). At the end of the study these dogs weighed 12.5±0.9 kg. Body weights were not significantly different between groups, although dogs consuming the low (n-3) FA food weighed significantly more after 36 weeks compared to baseline (P = 0.02).

### 3.2. Plasma FA profiles

The FA composition of plasma at the beginning of the study was similar for experimental dogs regardless of the projected dietary intervention (Table 2). The healthy Beagles used in the teaching program differed from the experimental dogs in total saturated fatty acids (SFA)(P<0.001), arachidonic acid (P<0.001), total polyunsaturated fatty acids (PUFAs) (P = 0.01), and total (n-6) FAs (P = 0.01). Plasma from these dogs had approximately 18% more SFA (primarily palmitic acid and to a lesser extent myristic acid) than experimental dogs,

and 18% less AA. This resulted in total PUFAs and total (n-6) FAs being approximately 8% less in these dogs compared to experimental dogs at baseline.

As expected, after feeding the two experimental foods for 36 weeks, plasma FA compositions changed substantially (Table 2). Sum of the (n-6) FAs, sum of the (n-3) FAs, and ratio of (n-6)–(n-3) FAs reflected the dietary composition of the respective FAs in the foods fed. Although plasma total (n-3) FAs ( $P<0.001$ ), including EPA ( $P<0.001$ ) and DHA ( $P<0.001$ ), were significantly higher in dogs fed fish-oil enriched food compared to dogs fed corn oil-enriched food, total (n-6) FAs ( $P<0.001$ ), including linoleic acid (LA) ( $P<0.001$ ) and AA ( $P<0.001$ ), were significantly lower such that

Table 2

Mean ( $\pm$ SEM) plasma concentration of fatty acids in geriatric Beagles before and after dietary intervention for 36 weeks with food that differed in the ratio of (n-6)–(n-3) fatty acids

Fatty Acid	Baseline food	Corn oil-enriched food with low (n-3) fatty acid content		Fish oil-enriched food with high (n-3) fatty acid content	
	4 weeks	Baseline	36 weeks	Baseline	36 weeks
14:0	0.9 $\pm$ 0.1 <sub>ax</sub>	0.1 $\pm$ 0.1 <sub>b</sub>	0.3 $\pm$ 0.1 <sub>y</sub>	0.5 $\pm$ 0.1 <sub>a</sub>	0.9 $\pm$ 0.2 <sub>x</sub>
16:0	16.6 $\pm$ 0.5 <sub>ax</sub>	9.4 $\pm$ 0.4 <sub>b</sub>	12.9 $\pm$ 0.5 <sub>y</sub>	11.0 $\pm$ 0.5 <sub>b</sub>	14.7 $\pm$ 1.0
18:0	20.4 $\pm$ 0.4	20.6 $\pm$ 0.8	20.4 $\pm$ 0.6	18.7 $\pm$ 0.6	19.6 $\pm$ 0.9
Sum of SFA <sup>c</sup>	38.6 $\pm$ 1.0 <sub>ax</sub>	32.5 $\pm$ 0.3 <sub>b</sub>	33.7 $\pm$ 0.6 <sub>y</sub>	32.9 $\pm$ 0.2 <sub>b</sub>	35.7 $\pm$ 1.1
18:1(n-9) <sub>c</sub>	9.3 $\pm$ 0.5 <sub>x</sub>	10.6 $\pm$ 0.5	8.0 $\pm$ 0.1 <sub>y</sub>	10.7 $\pm$ 0.4	7.9 $\pm$ 0.2 <sub>y</sub>
Sum of MUFA <sup>c</sup>	14.1 $\pm$ 0.6 <sub>x</sub>	15.8 $\pm$ 0.5	11.3 $\pm$ 0.4 <sub>y</sub>	15.8 $\pm$ 0.5	15.1 $\pm$ 0.6 <sub>x</sub>
18:2(n-6)	23.8 $\pm$ 0.8 <sub>x</sub>	22.9 $\pm$ 0.6	32.9 $\pm$ 1.2 <sub>y</sub>	22.8 $\pm$ 0.7	21.2 $\pm$ 1.3 <sub>x</sub>
20:4(n-6)	19.4 $\pm$ 0.7 <sub>ax</sub>	24.3 $\pm$ 0.7 <sub>b</sub>	19.4 $\pm$ 0.9 <sub>x</sub>	23.3 $\pm$ 0.3 <sub>b</sub>	11.8 $\pm$ 0.4 <sub>y</sub>
20:5(n-3)	0.2 $\pm$ 0.1 <sub>x</sub>	0.2 $\pm$ 0.1	0.1 $\pm$ 0.0 <sub>x</sub>	0.1 $\pm$ 0.1	6.6 $\pm$ 0.5 <sub>y</sub>
22:6(n-3)	0.6 $\pm$ 0.1 <sub>x</sub>	0.7 $\pm$ 0.1	0.4 $\pm$ 0.2 <sub>x</sub>	0.6 $\pm$ 0.1	4.3 $\pm$ 0.6 <sub>y</sub>
Sum of PUFA <sup>a</sup>	46.9 $\pm$ 1.4 <sub>ax</sub>	51.2 $\pm$ 0.7 <sub>b</sub>	54.9 $\pm$ 0.9 <sub>y</sub>	50.3 $\pm$ 0.4 <sub>b</sub>	49.1 $\pm$ 1.0 <sub>x</sub>
Sum of (n-6) fatty acids	44.0 $\pm$ 1.5 <sub>ax</sub>	48.4 $\pm$ 0.6 <sub>b</sub>	53.0 $\pm$ 1.3 <sub>y</sub>	47.7 $\pm$ 0.4 <sub>b</sub>	34.5 $\pm$ 1.2 <sub>z</sub>
Sum of (n-3) fatty acids	2.7 $\pm$ 0.2 <sub>x</sub>	2.9 $\pm$ 0.2	1.4 $\pm$ 0.2 <sub>x</sub>	2.6 $\pm$ 0.2	12.9 $\pm$ 1.4 <sub>y</sub>
Ratio (n-6)–(n-3)	16.4 $\pm$ 1.5 <sub>x</sub>	17.1 $\pm$ 0.9	40.5 $\pm$ 5.5 <sub>y</sub>	18.9 $\pm$ 1.7	2.9 $\pm$ 0.5 <sub>z</sub>

A separate group of healthy Beagles used in the College teaching program were fed baseline food for 4 weeks.

<sup>a</sup>Mean values with different letters are significantly different  $P<0.05$ , at baseline, before dietary intervention. <sup>b</sup>Mean values with different letters are significantly different  $P<0.05$ , after dietary intervention.

<sup>c</sup><sup>z</sup>See Table 1 for remainder of key.

total PUFAs ( $P=0.003$ ) were significantly lower after consumption of high (n-3) food. Plasma concentration of total monounsaturated fatty acids (MUFAs) ( $P=0.002$ ) was significantly higher in dogs fed fish oil-enriched food, whereas plasma concentrations of SFAs did not differ significantly between the dogs fed the high and low (n-3) FA foods.

The baseline food was not enhanced in (n-6) and (n-3) FAs compared to the experimental foods, and, thus after the experimental foods were fed for 36 weeks, the plasma ratio of (n-6)–(n-3) FAs in the healthy Beagles used in the teaching program (16.4:1) was intermediate between the plasma ratios of (n-6)–(n-3) FAs for the corn oil-fed dogs (40.5:1) and fish oil-fed dogs (2.9:1), and all were significantly different from each other ( $P<0.001$ ). Plasma total (n-3) FAs ( $P<0.001$ ), EPA ( $P<0.001$ ), and DHA ( $P<0.001$ ) were significantly higher in the dogs fed the fish oil-enriched food compared to dogs fed baseline food, but were similar in dogs fed the baseline food and the corn oil-



enriched food. Plasma total (n-6) FAs ( $P < 0.001$ ) and AA ( $P < 0.001$ ) in dogs fed the fish oil-enriched food were lower than those in dogs fed baseline food, but because EPA and DHA were increased in dogs fed the fish oil-enriched food, the total PUFA levels ( $P = 0.003$ ) were similar between the dogs fed baseline food and those fed fish oil-enriched food. Total PUFAs ( $P = 0.003$ ) were significantly higher in dogs fed corn oil-enriched food compared to dogs fed the baseline food, primarily because LA ( $P < 0.001$ ) (but not AA), and thus, total (n-6) FAs ( $P < 0.001$ ), were significantly higher in dogs fed corn oil-enriched food. Total SFAs ( $P = 0.02$ ) and total MUFAs ( $P = 0.002$ ) (including oleic acid [ $P = 0.02$ ]) were also lower in dogs fed the corn oil-enriched food compared to plasma levels in dogs fed baseline food. Total SFAs ( $P = 0.02$ ) and oleic acid ( $P = 0.02$ ) (but not total MUFAs) were lower in dogs fed the fish oil-enriched food compared to plasma levels in dogs fed baseline food.

### 3.3. LTB<sub>4</sub> and LTB<sub>5</sub> quantification

Neutrophils from dogs fed fish oil-enriched food produced 7.6-fold more LTB<sub>5</sub> ( $P = 0.002$ ) compared with dogs fed corn oil-enriched food (Fig. 1). No LTB<sub>5</sub> was detected in supernatants of stimulated neutrophils from dogs fed baseline food. Similar amounts of LTB<sub>4</sub> were produced by stimulated neutrophils from dogs in all three groups. The ratio of LTB<sub>5</sub> to LTB<sub>4</sub> concentrations was 8.3-fold higher ( $P < 0.001$ ) in dogs fed fish oil-enriched food compared with dogs fed corn oil-enriched food (0.50 vs. 0.06, respectively). The ratio of EPA to AA in the plasma across dietary treatments was correlated (correlation coefficient = 0.83,  $P < 0.001$ ) to the ratio of LTB<sub>5</sub>–LTB<sub>4</sub> concentrations.

Fig. 1. Effect of feeding geriatric Beagles foods for 36 weeks that differed in the (n-6) and (n-3) fatty acid content on the production of LTB<sub>5</sub> and LTB<sub>4</sub> by stimulated peripheral blood neutrophils. A separate group of healthy Beagles used in the College teaching program were fed baseline food for 4 weeks. Data are expressed as nanograms per  $1 \times 10^6$  cells (mean  $\pm$  SEM). Bars with P-values above them indicate when mean values differed significantly ( $P < 0.05$ ). Different lower case letters above bars indicate which groups are significantly different ( $P < 0.05$ ).

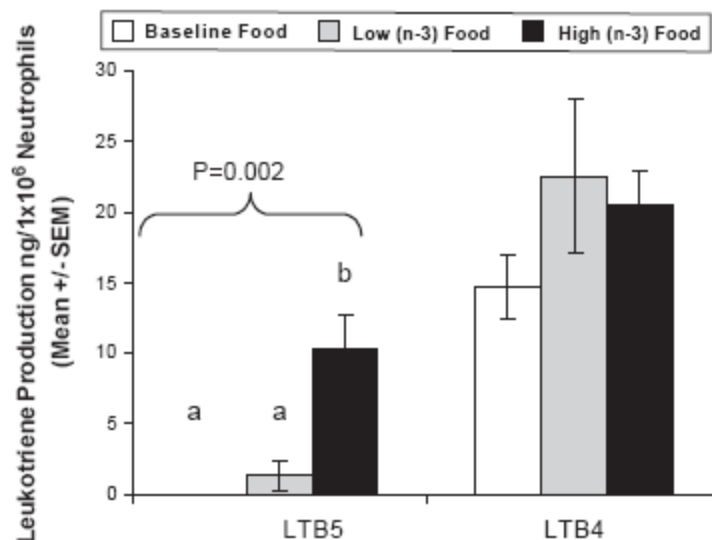


Fig. 1. Effect of feeding geriatric Beagles foods for 36 weeks that differed in the (n-6) and (n-3) fatty acid content on the production of LTB<sub>5</sub> and LTB<sub>4</sub> by stimulated peripheral blood neutrophils. A separate group of healthy Beagles used in the College teaching program were fed baseline food for 4 weeks. Data are expressed as nanograms per  $1 \times 10^6$  cells (mean  $\pm$  SEM). Bars with *P*-values above them indicate when mean values differed significantly ( $P < 0.05$ ). Different lower case letters above bars indicate which groups are significantly different ( $P < 0.05$ ).

#### 4. Discussion

The principal findings in this study were that production of LTB<sub>5</sub> and LTB<sub>4</sub> from canine peripheral blood neutrophils reflected the plasma concentration of substrates EPA and AA, respectively, from which they are derived. In turn, the plasma content of EPA and AA are dependent on the dietary concentrations of these FAs. Consumption of food enriched in fish oil resulted in higher plasma concentrations of EPA and DHA, and lower plasma concentrations of AA than consumption of food enriched in corn oil. Consequently, stimulated neutrophils from dogs consuming fish oil-enriched food produced higher levels of LTB<sub>5</sub>. Consumption of food enriched in corn oil resulted in higher plasma concentrations of LA, but not AA. There was no significant difference in the production of LTB<sub>4</sub> by stimulated neutrophils from dogs consuming food enriched in corn oil compared to dogs consuming food enriched in fish oil.

These results are similar in part to what has been previously reported for canine neutrophils. Experimental foods containing (n-6)–(n-3) ratios of 5:1, 10:1, 25:1, 50:1, and 100:1 were fed to Beagle dogs for 12 weeks [18]. Neutrophils from dogs fed 5:1 and 10:1 foods synthesized 370–500% greater LTB<sub>5</sub> and 30–33% less LTB<sub>4</sub> after 6 and 12 weeks of treatment feeding compared to 0 weeks. Our dogs fed fish oil-enriched foods produced 760% more LTB<sub>5</sub> compared with dogs fed corn oil-enriched foods. The decrease in LTB<sub>4</sub> production by neutrophils from dogs fed foods with low (n-6)–(n-3) FA ratios in their study is consistent with the decrease in plasma AA concentration that we found in our dogs fed fish oil-enriched food, but we report no statistically significant change in LTB<sub>4</sub> production after 36 weeks. It is possible that a small sample size and large variation between dogs for LTB<sub>4</sub> production prevented us from detecting a decrease in



LTB4 production in dogs fed fish oil-enriched food. Alternatively, even though AA content of plasma was significantly lower in dogs fed fish oil-enriched food compared to dogs fed corn oil-enriched food, perhaps it was not decreased enough to see a concomitant decrease in LTB4 synthesis by stimulated neutrophils. In humans, supplementation of the Western diet with icosapentaenoic acid for 4 weeks only slightly reduced AA content in human polymorphonuclear leukocytes, and consequently, there was unreduced synthesis of LTB4 by ionophore A23187 stimulated cells [19]. Our dogs fed fish oil-enriched food still received the same amount of (n-6) FAs/kg food as dogs fed baseline food. Our results in dogs also differ from our findings in horses [3], whereby feeding fish oil-supplements to horses results in an increase in plasma AA concentration and an increase in LTB4 production by stimulated neutrophils.

Similar to what we reported for horses [3], our dogs consuming a fish oil-enriched food showed a strong correlation (correlation coefficient = 0.83) between the ratio of EPA-AA in plasma and the ratio of LTB5-LTB4 synthesized by stimulated neutrophils. The ratio of plasma EPA-AA and the ratio of LTB5-LTB4 production were higher in dogs receiving the fish oil-enriched food compared with dogs receiving the corn oil-enriched food. If the ratio of LTB5-LTB4 is important in the dog, as it is in other species, then a diet high in (n-3) PUFA is preferable to a diet high in (n-6) PUFA in terms of decreasing proinflammatory leukotrienes synthesis. Others have shown a linear relationship between the ratio of EPA-AA in cell membrane phospholipids and the ratio of LTB5-LTB4 produced by rat peritoneal exudate cells in vitro [5]. In that study, the content of EPA in cell membrane phospholipids was positively correlated with LTB5 synthesis and negatively correlated with LTB4 synthesis. The content of AA in cell membrane phospholipids was positively correlated with LTB4 synthesis. Altogether, these results suggest that EPA and AA are equally active substrates for 5-lipoxygenase.

There is evidence that substrate concentration can enhance or inhibit production of eicosanoids [20–22]. EPA can act as a competitive inhibitor of AA conversion to LTB4 [23]. Using rat alveolar macrophages, Kobayashi et al. [21] found that EPA inhibited endogenous production of LTB4 from AA. In addition, LTB4 production decreased in a dose-dependent fashion when cells were incubated with EPA [22]. At low concentrations of EPA, generation of LTB5 was increased, whereas at high concentrations of EPA, generation of LTB5 was decreased. These authors suggested that at low concentrations EPA may compete with AA as a substrate, and that at high concentrations it may directly inhibit AA metabolism by inhibiting 5-lipoxygenase or phospholipase A2.

Results of our study suggest that a diet rich in (n-3) PUFA caused increased production of LTB5, which is less biologically active than LTB4, and may have inhibitory effects on the function of LTB4 [2]. Some studies suggest that the ratio of LTB5-LTB4 is more important than the absolute amounts of either eicosanoid [5,23–25]. For example, LTB5 inhibited the ability of LTB4 (which was present in optimal concentrations for activity) to stimulate DNA synthesis in cultured human epidermal keratinocytes, and inhibited chemotaxis of human blood neutrophils in a dose-dependent manner [24]. The authors

suggested that the inhibitory effect of LTB5 on LTB4 activity was related to competition for receptors.

Dietary treatment for 36 weeks resulted in altered plasma FA profiles, which were consistent with the respective supplement's FA profiles. Dogs consuming a fish oil-enriched food experienced an elevation of (n-3) FAs, predominantly EPA and DHA, and a decrease in (n-6) FAs, predominantly AA. Dogs were fed three times longer than in previous studies, demonstrating that changes in plasma FA profiles induced by short-term feeding (8–12 weeks) persist for longer periods of time as long as the dietary treatment is maintained.

This study supports the hypothesis that feeding fish oil results in partial replacement of AA in cell membranes by EPA, which in turn leads to decreased production of AA-derived eicosanoids. This may result from decreased availability of AA, competition of AA and EPA for lipoxygenase enzymes, and/or decreased expression of 5-lipoxygenase, as LTB4 is a product of the 5-lipoxygenase pathway of AA metabolism. This would explain in part the anti-inflammatory effects of (n-3) FAs [10]. Alterations in leukotriene expression may also lead to altered gene expression through effects on transcription factor activation, such as nuclear factor- $\kappa$ B (NF- $\kappa$ B) [2,8,26]. It is known that dietary fish oil results in suppressed production of proinflammatory cytokines [8]. Recently, LTB4 was shown to increase monocyte chemoattractant protein-1 expression, a proinflammatory cytokine, and this increase was mediated via NF- $\kappa$ B [9]. LA has been shown to increase secretion of IL-8 by Crohn's human intestinal smooth muscle cells by stimulating production of AA metabolites (LTB4, PGE2, and thromboxane B2 [TXB2]) [27]. Inhibition of 5-lipoxygenase (and thus production of LTB4) blocks IL-8 production. It is proposed that LTB4 increases IL-8 production by enhancing NF- $\kappa$ B-dependent transcription of IL-8 [27]. Thus, neutrophils secreting LTB4 may promote production of a transcription factor, NF- $\kappa$ B, which leads to expression of proinflammatory cytokines.

In summary, we have shown that enrichment of foods with fish oil increased production of the less inflammatory eicosanoid LTB5 in dogs. The ratio of plasma EPA–AA corresponded with the ratio of LTB5–LTB4 produced by stimulated canine neutrophils. Further studies are needed to determine the optimum ratio of LTB5–LTB4 concentrations in canine inflammatory disorders, e.g., inflammatory bowel disease and pruritic dermatological conditions such as atopic dermatitis, for decreasing activation of NF- $\kappa$ B and subsequent production of proinflammatory cytokines.

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